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CORRELATION OF TENSILE STRENGTH AND CHEMICAL COMPOSITION IN EXPERIMENTAL GRANULOMA

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The experimental granuloma has been the subject of numerous chemical studies (for general references see *Woessner & Boucek,* 1961) but it has not been possible to compare directly the tensile strength of the recently formed collagenous fibres with the chemical composition of the granulation tissue. From a healing wound the granulation tissue is difficult to prepare quantitatively for chemical study from the adjoining original tissue and it has not been possible to estimate the tensile strength of whole pieces of experimental granulomata.

The emphasis of the present investigation was on the direct comparison of the chemical and mechanical properties of the same granulation tissue. The principle was to use pieces of suitable implantation material, which had been divided into two halves but fixed again in original position with stitches. After the implantation period in subcutaneous space of the rat, the experimental granuloma could be separated clearly from the surrounding tissue. The stitches were removed and the tensile strength measured by drawing the two halves apart. The second point of emphasis was to study the immediate period after implantation, *i.e.,* the "lag phase" before the collagen synthesis begins. Into this methodical paper we included also the effect of immersion of the implantation materials into solution of soluble collagen, and into solution of carrageenin, which has frequently been used to provoke the formation of experimental granulomata (*Robertson & Schwartz,* 1953).

EXPERIMENTAL

Animals. Wistar rats (about 100 g) were used. At this age the necessary space for the implantation material could be prepared with a minimal traumatization of

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the subcutaneous tissue. In later experiments it was found that the age and the weight of the animals somewhat influence the results. The animals were kept in wire net cages, usually 2 in each.

Technique of implantation. The animals were anaesthetized with ether. The shaved skin was cleaned with ethanol and about 3 cm long medial incision was done on the back caudally of the scapula. The space between fascia and skin (with subcutaneous fat) was opened with blunt-ended forceps and two implants were placed symmetrically. The incision was closed with sterilized cotton yarn.

Implantation materials. In the earlier experiments "Sponcal"–sponge (viscose cellulose) tampons (standard pieces of about 40 × 10 × 10 mm wet, manufactured for the dental practice by Svenska Cellulosa Ab., Sundsvall, Sverige) were used. The pieces of the range 170-190 mg were used, but cut 20 mm long. Later we used pieces of viscose cellulose sponge "Visella" (prepared for us by Säteri Oy. Ltd. Valkeakoski, Finland). The sulphur had been removed by washing, the pieces bleached and finally treated with glycerol to soften them. One cm^3 of dry Visella sponge weighed 74 mg. The average pore size ("open cell system") was about 0.25 mm diameter, and the combined surface area of the pores about 60 cm^2/cm^3 sponge. Most of the present experiments were made with "Sponcal"-sponge; only in the "regranulation" experiments "Visella"-sponge was used. However, the "Visella"-sponge was adopted to the standard use. As far we know, the basic material is the same both in "Sponcal" and in "Visella", but in the latter the pore size is smaller, which is an advantage. The granulation tissue grows into it easily, better than into "Sponcal"-tampons. We appreciate the advantages of the viscose cellulose as implantation material: it is rather homogenous, easy to cut and stitch, inert and thus suitable for mechanical and chemical study, sterilizable by dry heat or boiling and easily impregnable with desired solutions.

The pieces 20 × 10 × 10 mm were cut with scissors in two halves which were sewn together with 4 stitches. They were sterilized in boiling water for 15 min.

In one experiment the pieces were immersed in sterile carrageenin solution (in 0.9 per cent NaCl). Selected leaves of Irish moss (purchased from Tampereen Rohdos Oy., labelled "Carragen tot", had been minced in a mortar, and the carrageenin was extracted for one hour at +90° C with 100-fold (v/w) volume of 0.9 per cent NaCl-solution. The viscous solution was clarified by centrifugation (20 min., 4300 r.p.m.).

In an additional series the implanted material was soaked with neutral salt-soluble collagen solution, prepared from rat tail tendons. Rat tail tendon fibres were prepared from rats of about 150 g weight and extracted immediately with 1*M* NaCl-solution at +5° C overnight. The viscous solution, which contained about 1.5 mg collagen/ml, was cleared by centrifugation (about 30 min., 4300 r.p.m.). The sterilized dry sponge was imbibed with this solution at +5° C.

In the "regranulation" experiment the pieces were first implanted for 5 days, taken out and the halves drawn apart without removing the through-going yarns, which kept the halves together. Then the halves were allowed to take the original position and the pieces were implanted again for further 7 days, after which the tensile strength was measured.

Measurement of the tensile strength. The tensile strength was measured with a triple beam balance, nominal sensitivity 1 mg. After removing the stitches the implanted double piece was suspended to one side of the balance from the upper half and the lower half of the sample was fixed. A beaker was put on the other side of the balance. This side of the balance was now charged by allowing water to flow (350 ml/min.) into the beaker until on the other side the halves of the implanted sponge broke apart. The weight of the water was recorded. The skin wounds were tested similarly from 1 cm broad strips, excised perpendicularly to the wound.

Chemical determinations. For chemical determinations the implanted sponges were dried for 4 days at +80° C, weighed and homogenized with "Bühler" homogenizer to 2.5-fold (v/w) volume of fluid at full speed (nominally 50.000 r.p.m.). The collagen was gelatinized in autoclave (2 atm., 2 hrs, with water in neutral pH) and filtered through sintered glass (corresponding to Jena 11 G 4) which retained all the insoluble cellulose. It was observed that when implantation lasted longer than 2 days, some collagen (less than 10 per cent of the total) was still present in the residue and had to be measured separately. Total nitrogen was analyzed by

Kjeldahl combustion and subsequent destillation of ammonia. The collagen content was calculated from the hydroxyproline content determined according to *Neuman & Logan* (1950).

The samples for nucleic acid determinations were stored at —15° C. To 0.2 ml of the tissue homogenate (in 0.9 per cent NaCl) 0.2 ml of 10 per cent trichloroacetic acid solution was added and heated 15 min. at +90° C. The suspension was diluted with 2 ml of 5 per cent trichloroacetic acid. The mixture was filtered and the precipitate washed with 2 ml of 5 per cent trichloroacetic acid. The ultraviolet light absorption was measured from the combined supernatant at 2600 Å with Beckman DU spectrophotometer against reagent blank solutions. The total amount of nucleic acid was estimated from this absorption using a standard curve prepared from "Deoxyribonucleic Acid" (ex herring sperm; L. Light & Co. Ltd., Colnbrook, Bucks, England). The DNA was measured with diphenylamine method (*Burton* 1956). From the total value of the nucleic acid the amount of DNA was subtracted to get the estimate of RNA. Similar method was used by *Biggers, Lawson, Lucy & Webb* (1961) in their study on developing cartilage. Our results are of the same magnitude as reported by *Biggers et al.* (1961) and *Williamson & Guschlbauer* (1961), but the RNA values may be too high because of the error caused by protein impurities.

RESULTS

Morphological description. The paraffin blocks were sectioned at 10 μ and stained with haematoxylin-eosin and Heidenhain's azan. After 24 hrs. implantation the sponge was surrounded by a thin capsule (staining faintly blue with azan) and a zone of mononuclear leukocytes. The cells invaded to the center of the sponge during the second day. On the third day the first elongated fibroblasts and erythrocytes were observed, but distinct capillaries first on the fourth day. More fibroblast "islets" appeared and their outgrowths stained pale blue with azan. The fibrin retired gradually. From the seventh day onwards the fibroblasts increased, filled the entire sponge and the azan staining turned more intense. However, the change in the histological structure did not clearly reflect the abrupt increase of the tensile strength and collagen (Fig. 2). Histological pictures of the sponge granulomata are published in other context (*Viljanto & Kivikoski* 1962).

DNA, RNA and total nitrogen. The data (Fig. 1) show in agreement with *Williamson & Guschlbauer* (1961) that 2–5 times RNA is produced in comparison to DNA, which latter reaches the maximum at 7th day. After the very first days a temporary decrease in RNA is noted. The total nitrogen increased earlier than collagen. We believe that this initial increase of nitrogen is not primarily due to increased intracellular protein but to imbibition of the pieces with tissue fluid and from 3 days onwards with blood carried through the developing capillaries (cf. *Jackson* 1958).

Tensile strength and amount of collagen. Fig. 2 summarizes the results. About 2–3 days after implantation there is a first slight maximum in collagen content. From the 5th day onwards the main collagen synthesis begins, which is parallelled with the development of the tensile strength.

The effect of carrageenin. In Table 1 it is shown that the soaking of the sponge in carrageenin does not have any beneficial effect on the

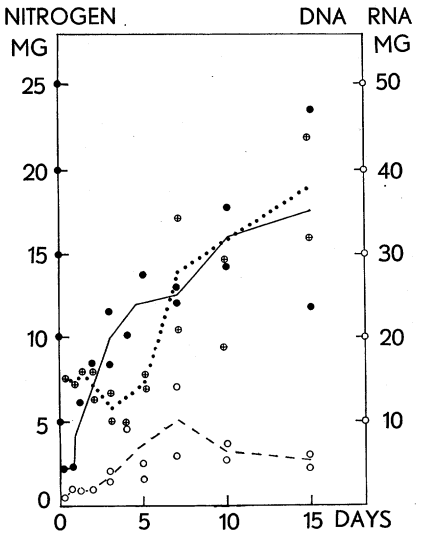

Fig. 1.

DNA (– – ○ – – ○ – – ○ – –), RNA (•• ⊕ •• ⊕ •• ⊕ ••) and total nitrogen (—●—●—) per piece of "Sponcal"-granuloma tissue.

The lines are fitted visually. At the time range 6–48 hrs. eight granuloma pieces were homogenized together into one sample. At later time points the granulomata from 4–5 rats were combined to one homogenate. Each point represents one homogenate.

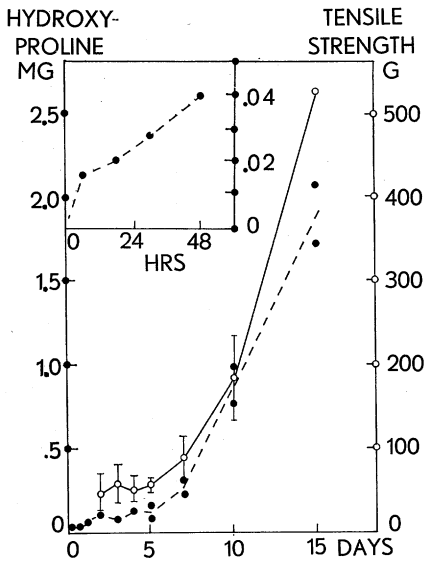

Fig. 2.

Tensile strength (—○—○—○— with standard deviation) and hydroxyproline (– –●– –●– –●– –) per piece of "Sponcal"-granuloma tissue.

The hydroxyproline content during the first 48 hrs. is shown in the insert. Each point of hydroxyproline represents one homogenate of eight granuloma pieces from 4 rats.

TABLE 1

Effect of Carrageenin on the Tensile Strength of Granuloma Tissue

Days implanted	No. of implants	Average tensile strength, g/piece		
		Control	Carrageenin	$\frac{\text{Carrageenin}}{\text{Control}} \times 100$
2	2	40.2	20.9	51.9
3	3	39.4	33.7	85.5
4	3	41.9	43.5	103.8
5	3	56.3	35.9	63.7
7	3	42.4	34.5	81.3
15	2	129.7	106.1	81.8
				Average 78.0 ± 18

tensile strength of the granulation tissue. Carrageenin accordingly acts as a foreign body only and seems not to have any specific stimulating effect on the collagenous tissue. This finding is at variance with the experiments of *Bush & Alexander* (1960) who used cotton pellets as implantation material and enchanged the granuloma formation by

soaking the implants with carrageenin solution. With cotton pellets we found some benefit from carrageenin 4 days after implantation on the weight of the granulation tissue. The sample of carrageenin had been used successfully in the production of granulomata. In some experiments, not described in detail, the fractions of carrageenin (prepared according to *Smith, O'Neill & Perlin,* 1955) were tried (without sponge) and the λ-fraction was more active than κ-fraction, but the difference was not marked.

TABLE 2

Effect of Neutral Salt-Soluble Collagen on the Tensile Strength of Experimental Granulation Tissue.

Days implanted	Tensile strength, g/piece	
	Control implants	Implants imbibed with soluble collagen
1	8.6	11
2	40	61
3	47	58
5	49	62
7	73	83

Each figure is the average of three determinations. The difference between the groups is statistically significant ($P < 0.005$), when tested as non-independent pairs.

TABLE 3

Tensile Strength of Experimental Granulation Tissue and Skin Wounds after Preliminary Implantation of the Viscose Cellulose Pieces.

Experiment	Tensile strength (g) on 7th day	
	Granuloma	Skin wound
After 5 days preimplantation	86 ± 19 (4)	350 ± 96 (5)
Controls	48 ± 25 (5)	201 ± 69 (8)

The standard deviation is indicated. Number of measurements in the parentheses.

Effect of soluble collagen and "regranulation". Table 2 shows that the tensile strength was increased when the implants had been impregnated beforehand with soluble rat collagen ($P < 0.005$). If granulation tissue is present in the sponge already on the "zero day", the tensile strength develops much stronger at continued implantation than without preexisting granulation tissue (Table 3).

DISCUSSION

This procedure has certain advantages: (1) The tensile strength can be measured on a well defined granulation tissue, which is grown in (2) sterile conditions. This arrangement is decidedly better for the production of granulation tissue than open skin wounds, which are often infected and their granulation tissue is not easily delimited. (3) The

volume of the granulation tissue is kept constant, at least during the early phase. An advantage over turpentine abscesses is that (4) the irritation of the surrounding tissue is minimal. There is also a (5) possibility to imbibition of the granulation tissue with desired solutions. However, it was observed that the granulation tissue develops stronger near the surface of the sponge and therefore it is important that the circumference of the cross-sectioned area (of contact between the halves) is constant and not too small. Perhaps better results would be obtained with a cross-sectional area of a parallelogram. The standard deviations of various groups, in average 8 measurements, were calculated in per cent and these standard deviations in the tensile strengths of "Visella" granulomata were in the range of 4.8–35.4 per cent (17 groups, average 22 per cent), in skin wounds 5.8–33.3 per cent (17 groups, average 24 per cent).

The origin of the collagen during the first days is obscure. Either a cellular source may be taken into consideration or it may be suggested that the imbibing tissue fluid contains some soluble collagen. At the next phase the amount of collagen rather decreases during the proliferation and maturation of the fibroblasts before the final main collagen synthesis begins. The next step of this investigation is the elucidation of the factors, which determine the consequent details in collagen synthesis: amino acids, soluble collagens, maturation to insoluble fibres and the balance with simultaneous decomposition of collagen.

The rather modest effect of soluble collagen could be explained most easily by a clot formation. It has been found by several workers that fibrin clot inside the sponge implant enchanges the formation of granulation tissue (*e.g., Edwards, Sarmenta & Hass,* 1960). Thus it is not necessary to consider any specific effect of added soluble collagen on the formation of fibres. *Peacock* (1961) made an extensive study on the effect of the collagenous extracts on wound healing. He got positive results with acid-extracted (but neutralized) collagen solutions only. He concludes that "if collagen from external source has any opportunity to participate in the formation of new scar tissue, it will probably have to be introduced as a monomeric form".

From the Table 3 it is obvious that when a ready granulation tissue is present, the "lag phase" before the collagen fibre formation is shortened. It is not known whether intact granulation tissue is necessary or whether *e.g.,* homogenate would be sufficient. The faster healing of a resutured wound has been often cited in the literature (for general reference see *Weiber* 1961).

SUMMARY

The growth of granulation tissue into viscose cellulose sponge was studied with emphasis (1) on the comparison of the tensile strength and chemical composition of the granulation tissue and (2) on the "lag phase" immediately after implantation.

Collagen is observed in experimental granuloma in small scale in few hours after implantation, and significantly from the fifth day onwards. The tensile strength runs approximately parallel.

Soaking of the implanted material with soluble collagen increased the tensile strength of the granulation tissue. Imbibition with carrageenin slightly decreased the tensile strength of cellulose sponge granulomata.

The total nitrogen in the implanted sponges increased very rapidly, presumably through the imbibition with tissue fluids and blood. Preliminary data are presented on the formation of DNA and RNA.

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